SURFACE MODIFICATION FOR BIOCOMPATIBILITY

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FIGURE CAPTIONS

Figure 1: Change in 13F signal over time in PBS only or PBS/0.001% BSA. The data for BPS/0.001% BSA are represented using a more accurate model for the adsorbed protein layer to account for the reduction of the XPS signal by that overlayer.

Figure 2: Changes in ion concentrations over time indicate glass dissolution.

Figure 3: Phase contrast photomicrographs showing cultures of embryonic day 22 rat cerebral cortex at 13 days post-plating on the substrates Poly-D-lysine and 13F. Cultures were maintained in MEM+ N3/g containing one of the following growth factors: CNTF (5 ng/ml), bFGF (20ng/ml), BDNF (100 ng/ml). BDNF was found to contribute to longevity of neuronal cells. bFGF was found to enhance survival, but did not contribute as significantly to neuronal longevity.

Figure 4: Phase contrast photomicrographs showing cultures of post-natal day 10 rat cerebral cortex at 24 hrs. post-plating on Poly-D-lysine and OTS. These cells were maintained in MEM + N3/g with or without 20ng/ml bFGF. The presence of bFGF enhances cell outgrowth and attachment on both substrates.

Figure 5: Phase contrast photomicrographs showing cultures of embryonic day 14 rat cerebral cortex at 4 days post-plating on the substrates OTS and 13F. Cultures were maintained in MFM+N3/g with or without 20 ng/ml bFGF. The presence of bFGF enhanced cell outgrowth and attachment on these "poorer" surfaces.

Figure 6: Phase contrast (1,3,5) and fluorescence (2,4,6) photomicrographs showing enriched cultures of rat microglia derived from the cerebral cortex of post-natal day 2 pups. Enriched microglial cultures were obtained by subjecting 2 week old mixed microglial-astrocyte cultures to 12 hr. of shaking on an orbital shaker. The microglia were collected by centrifugation and seeded on Poly-d-lysine (1 - 4) and DETA (5,6) coated coverslips and maintained for two weeks. Microglia were immunocytochemically identified with OX 42 (1:100, Serotech) antibody. Most of labeled cells displayed an ameboid form (2,4,6) and a few OX-42 positive cells developed long, thin processes (4). Oligodendrocytes (arrow, 1) and the astrocytes (arrow, 5) were unlabelled.

PROJECT SUMMARY

The aim of this work is to create surfaces on implantable silicon microstructures for the purpose of controlling the interaction of neurons, glia, and related cells and their protein products with the microstructure. The third and fourth quarters were primarily devoted to the examination of culture conditions for early embryonic cultures with an aim to promote longevity in culture. We established E16 cortical cultures which survived to 21 days. E19 to 19 days. As presented in earlier reports, we established a rudimentary serum-free culture to approximate the composition or cerebral spinal fluid (CSF) and have extended screened artificial surfaces to: two (2) surfaces for E 14 response; nine (9) surfaces for E 16 cortical cell response, thirteen (13) surfaces for E19 cortical cell response; 6 surfaces for E22 cortical cell response; two (2) surfaces for post-natal day 10 response; five (5) surfaces for glial astrocyte cell response, and two (2) surfaces for microglial response.

This quarter we primarily focused on later embryonic (E22) cultures; and post-natal (PN10) cultures in which we initiated a preliminary screening of substrates and examined procedures most suited to establishment of long-term survival of these more challenging preparations. Experiments for culture of E14 embryonic tissue and post-natal day 2 microglia were also initiated.

Continuing surface stability experiments indicate that at least some of the artificial surfaces (silane monolayers on glass) are stable for much longer times in the presence of proteins, but the glass we used, which is porous, begins to dissolve after four to eight weeks. We are extending these experiments to other substrates such as polyethylene and also to see the effect of increasing the protein concentration 10x.

In addition to the previously examined SAM surfaces, we are in the process of researching and producing some novel SAMs, modified with crosslinkers which will allow the attachment of selected biological macromolecules, for testing in culture. In particular, a two component SAM (mixed monolayer) was prepared from 3-mercaptopropyltrimethoxysilane and n-(2-aminoethyl)-3-aminopropyltrimethoxysilane to isolate amine functional groups from each other. A homobifunctional cross linker such as diethyl malonimidate can be reacted under basic conditions with these amine

functionalities leaving one reactive imidate on the surface for reaction to any terminal amine from a biological protein such as BSA or laminin.

OBJECTIVES

Overall project objectives:

a) Selecting candidate surfaces that are likely to enhance the microscopic mechanical stabilization of a microstructure implanted within the central nervous system;

b) Selecting candidate organic surfaces that are likely to enhance the close approximation of neurons or neuronal processes to specific regions of implanted silicon microstructures;

c) Developing or adapting available methods to bond the selected organic molecules to a silicon dioxide surface like the surface of a micromachined electrode (Tanghe and Wise, 1992) and to chemically characterize these surfaces before and after protein adsorption.

   1. The attachment method shall be stable in saline at 37°C for at least 3 months;
   2. To use silane coupling as the method of attachment;
   3. To use the silanes to control the spatial extent (i.e., the pattern) of the deposited surface.

d) Developing a cell culture or other suitable model of mammalian cortex and investigate the growth and adhesion of neurons, glia, micro-glia, and other cells present in the nervous system on substrates coated with the selected surfaces;

e) Cooperating with other investigators in the Neural Prosthesis Program by coating microelectrodes (estimated 50 over the contract period) with the most promising materials for *in vivo* evaluation as directed by the NINDS Project Officer.

<u>QUARTER OBJECTIVES</u>

- Continue working to establish cortical cell culture conditions for optimal 4 week survival

- Begin screening biologically modified SAM surfaces for cortical cell survival
- Continue screening E22 response to artificial surfaces
- Continue surface analysis of surfaces both before and after culture
- Continue surface stability experiments in saline + BSA at 37°C
- Finish screening surface for glial response to non-ideal SAM surfaces
- Continue screening non-ideal surfaces for microglia response
- Begin PN10 culture experiments
- Send more samples to Huntington to continue *in vivo* experiments

BACKGROUND

Biomaterials that penetrate into the central nervous system as the microscopic electrode shafts of neural prostheses interact with neural and other tissues on a cellular and molecular level. In order to achieve tight coupling between these implanted microelectrodes and the target neural substrate, this interaction must be understood and controlled. Controlling the interaction requires an understanding of how cells, including neurons and glia, and extracellular proteins respond to the surface chemistry and any leachable substances of implanted biomaterials. This contract supported research will study these interactions with a long-term goal of rationally designing microelectrode surfaces to promote specific tissue interactions.

Presently, available clinical neural prosthetic implants typically use stimulus levels that excite volumes of neural tissue ranging from cubic millimeters to cubic centimeters around the electrode. Because of the large stimulus intensities required, precise control of the response of neurons within the first few cell layers of an implanted electrode has not been necessary. Recent developments in the areas of micromachining and fabrication of silicon integrated circuit microelectrodes have introduced the possibility of controlled stimulation of smaller volumes of neural tissue--on the order of one thousand to one hundred thousand times smaller than those used today.

The efficiency of these microelectrodes depends on the micro-environment around stimulating sites. The surface of the microelectrodes and the proteins that adsorb to this surface have a major impact on the way in which different cell populations react to the

implant. Neural growth cones are sent out from many neurons around a microelectrode following implantation. With appropriate surfaces it may be possible to get selected neurons to send processes directly to the microelectrodes. Glia and other cells also respond to an implanted electrode. With appropriate surfaces it may be possible to promote cell adhesion and anchoring of some areas of the implant structure while leaving other areas with minimal response from glial cells. This study will investigate cellular and molecular responses to specific surface modifications of silicon microelectrodes.

RESULTS

Surface Analysis and Stability Measurements

X-ray photoelectron spectroscopy (XPS) is necessary for this program in the same way that an NMR spectrometer is necessary for conducting an organic synthesis program. Since we are synthesizing surfaces and modifying their properties, we will need to assay the result of the surface before (starting material) and after (reaction product) modification. This is analogous to examining a procedure by NMR. One would not think to run an organic synthetic reaction with only an occasional examination of the actual product; in the same sense, it is crucial for us to examine the product in our surface modification experiments.

Our results to date indicate that the silane monolayers are much more stable in the presence of protein than in PBS only. Figure 1 compares the 13F signal (F 1s) of slips in PBS alone versus PBS with 0.001% BSA, using a more accurate model for the adsorbed protein layer to account for the reduction of XPS signal by that overlayer. After 4-8 weeks, we see a rapid decrease in the F signal but also the appearance of ions (Ti, Na,...) that are indicative of the glass dissolving (Figure 2). This makes sense in that the coverslip glass is porous. This is a significant result not only for implants but for our long-term culture results as well. To further investigate this we will repeat the experiment with a non-porous SiO_2 (SiO_2/Si on a wafer) as well as with a polymer that does not dissolve in PBS

(polyethylene). We will also determine how increasing the protein concentration affects this process.

Cell Culture

As illustrated in previous reports, we have developed of a rudimentary serum-free culture system for the cortical cells to more closely approximate the composition of cerebral spinal fluid (CSF). In the fourth quarter we concentrated on defining and examining the culture conditions to optimize longevity for both cortical and astrocyte cultures. In this capacity, we evaluated various tissue culture conditions (such as medium, additives, neurotrophic factors) which could play important roles in cell survival and differentiation.

This quarter we concentrated our examinations of tissue culture conditions primarily to additives, neurotrophic factors and developed methods and media that would support later embryonic (E22) and post-natal cultures (PN10).

We initially conducted the both the dissociation of E22 and post-natal day 10 cortical tissues utilizing a mechanical dissociation/ trituration protocol used for earlier embryonic cultures, because it is our standard protocol and a necessary control. With the increase in embryonic day (continuing into post natal days and adulthood) there are concomitant increases in dendritic arborization and axonal length in cortical cells and increases in contaminating debris from white matter and glial cells. As expected, the mechanical dissociation resulted in low yields and significant cell damage. We modified our standard protocol which, while using some of the same basic components, included the addition of the enzyme, papain (Schaffner, et al. 1995) and limited the mechanical treatment to tissue excision and coarse subsectioning/slices rather than trituration via pipette. Papain treatment with limited mechanical intervention resulted in increased cortical cell yield and survival.

We selected various neurotrophic factors suggested by the literature to promote survival of earlier embryonic cortical neurons in culture. In our preliminary studies of E22, BDNF, bFGF and CNTF were shown to promote survival of cortical neurons in culture (Ghosh, et al., 1994; Jones, et al., 1994; reviewed by Lempe and Qian, 1995; Arakawa et al., 1990).

Figure 3 illustrates cultures of embryonic day 22 rat cerebral cortex at 13 days post-plating. Cultures were maintained in MEM+ N3/g containing one of the following growth factors: CNTF (5 ng/ml), bFGF (20 ng/ml), BDNF (100 ng/ml). While the addition of each of these growth factors facilitated survival, as compared to the control, only BDNF was found to significantly contribute to longevity of the E22 cultures. bFGF was found to promote early growth, but it was less effective with regard to cortical neurons at later time points.

This latter finding of neurotrophic factors contributing to cell survival and longevity at different times has some particular significance with regard to the birthdates of cells taken from different embryonic stages and maintained in culture. We have been able to maintain E16 cultures for 21 days, E18/19 cultures for 19 days, and now E22 for 13 days. Taken in the context of birthdates, under similar culture conditions, there is some predictability with regard to survival based on date of birth. The next challenge is extending these times to 4 weeks for E 16, and the subsequent survival dates, longevities for each of the other embryonic days examined.

It is known *in vivo* factors involved in cell growth and maturity change with development. These temporal changes in cell requirements could be mirrored in culture, and in our that early outgrowth is well supported by the addition of bFGF (Figure 4, (PNd10); Figure 5, (E14), E22 (not shown) but longevity is more readily supported by BDNF (Figure 3, (E22); (E18/19) previous report). One of our goals in the next quarter is to extend survival of embryonic and post-natal cultures and to find the potential role that different neurotrophic factors play at different times in development.

In addition to beginning post-natal day 10 cultures and extending our understanding of E 22 cultures, we also continued our examination of astrocyte cultures and initiated microglial cultures.

Mixed glial cultures were prepared from 2 day old neonatal rat. Briefly, cerebral cortices were removed and the meninges were stripped away and the tissue triturated, pelleted, and plated in Dulbecco's Modified Eagle's Medium (DMEM) + 10% fetal calf serum with glutamine, glucose, gentamycin and fungizone additives (Gibco). After 14 days in cultures, the flasks were shaken for 12 hr. at 37°C at 250 rpm. The microglia were collected from the solution, pelleted, and initially plated for 40 min. in either serum

containing or serum-free medium, prior to addition of a final serum-free medium HL-1 (Hicor). In this initial culture we determined the best conditions for initial attachment on SAM substrates, DETA and 13F. Other researchers, utilizing biological substrata, often find adhesion is enhanced with an initial attachment treatment with serum (Colton, personal comm.). We found adhesion and survival were greater with an initial plating in serum free conditions (vs. serum), in keeping with the stated goal of developing an *in vitro* system mirroring the conditions found *in vivo*. Purity was determined after two weeks with staining with OX-42 (1:100, Serotech) antibody specific for microglia (Figure 6).

Microglia are present two basic morphologies, consistent with their function/state of activation (ramified and ameboid). Our cultures primarily displayed the ameboid morphology (Figure 6, panel 2,4,6) with some few cells producing processes (Figure 6, panel 4), but do not resemble structures found in ramified microglia, *in vivo* . These cultures were approximately 70% pure, with some contaminating oligodendrocytes, and astrocytes (Figure 6, panels 1,5).

Collaborations

We have established a collaboration with W. Agnew at the Huntington Medical Research Institute. While we have not yet specifically modified electrodes, we have successfully modified the poly-silastic sheath that holds a series of microelectrodes in place along the spinal cord during implantation (Agnew et al., 1990). One problem we are focused on solving is that the sheath has glial scar buildup and adhesions that eventually displace the electrodes laterally (with adhesion) and vertically (due to buildup of tissue). We visited Huntington Institute after the Neuroscience meeting in November, and have planned a series of experiments. We will begin a new series of experiments once the costs of the *in vivo* work is determined. We are also examining by surface analysis some postmortem sample supplied by D. Agnew's group. These results will be reported later.

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NEXT QUARTER OBJECTIVES

- Continue work to establish cortical cell culture conditions for optimal 4 week survival
- Begin screening biologically modified SAM surfaces for cortical cell survival
- Continue screening E14 response to artificial surfaces
- Finish screening E22 response to artificial surface
- Continue screening PN10 response to artificial surfaces
- Continue surface analysis of surfaces both before and after culture
- Continue surface stability experiments in saline
- Continue screening surfaces for microglia response
- Send samples to Huntington to continue *in vivo* experiments as well as explore other possibilities for collaboration in this area.

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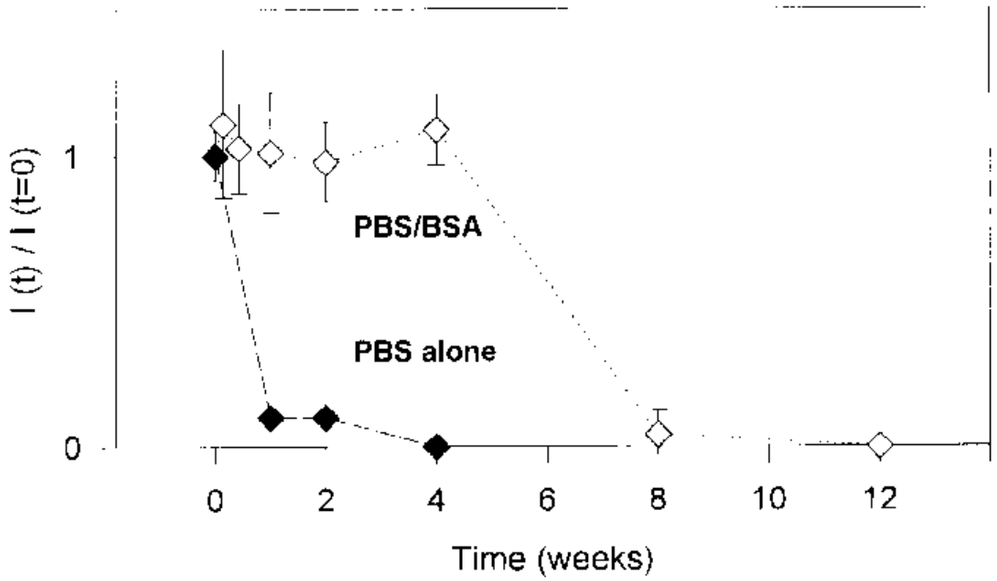
I (t) / I (t=0)
1
0
PBS/BSA
PBS alone
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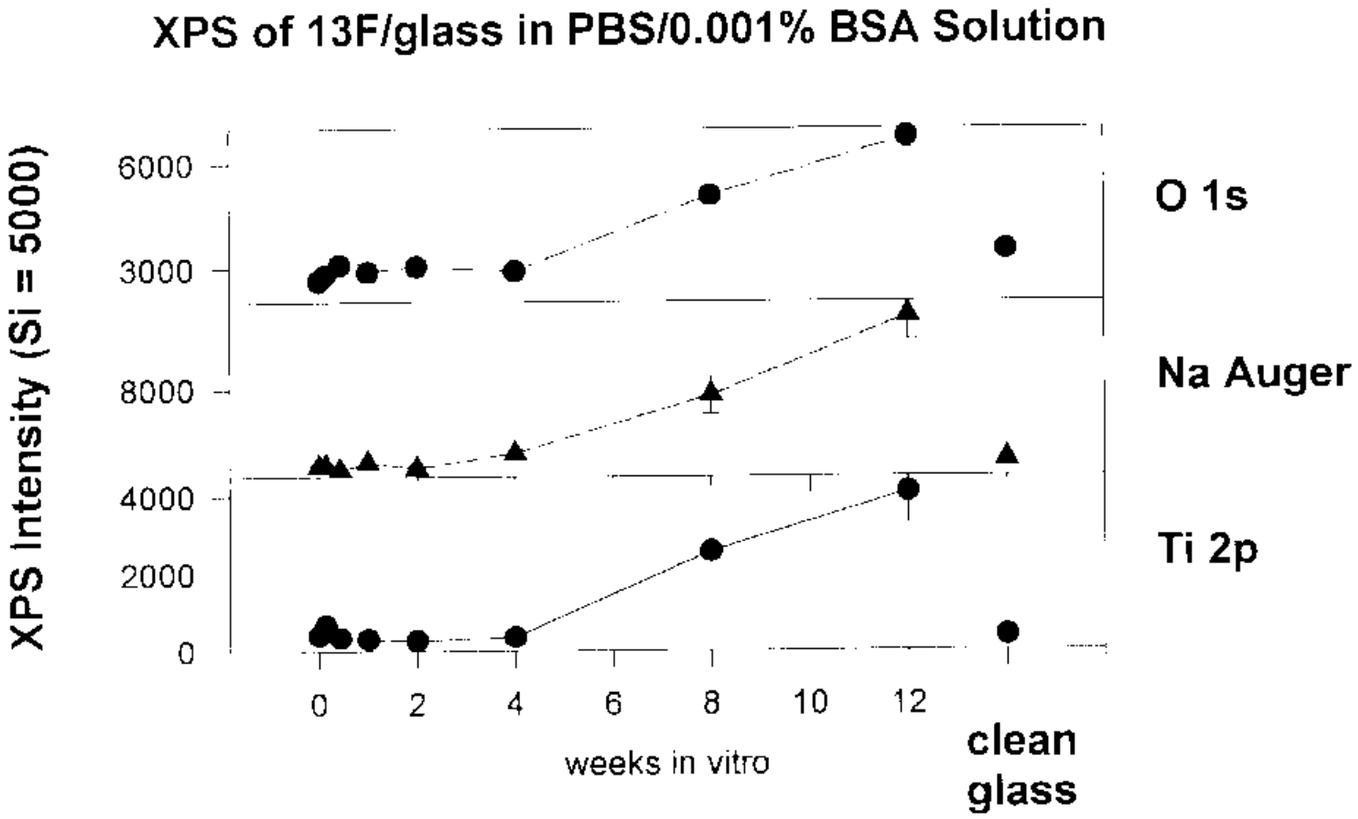
2
4
6
8
10
12
Time (weeks)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3000
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4000
2000
0
0
2
4
6
8
10
12
weeks in vitro
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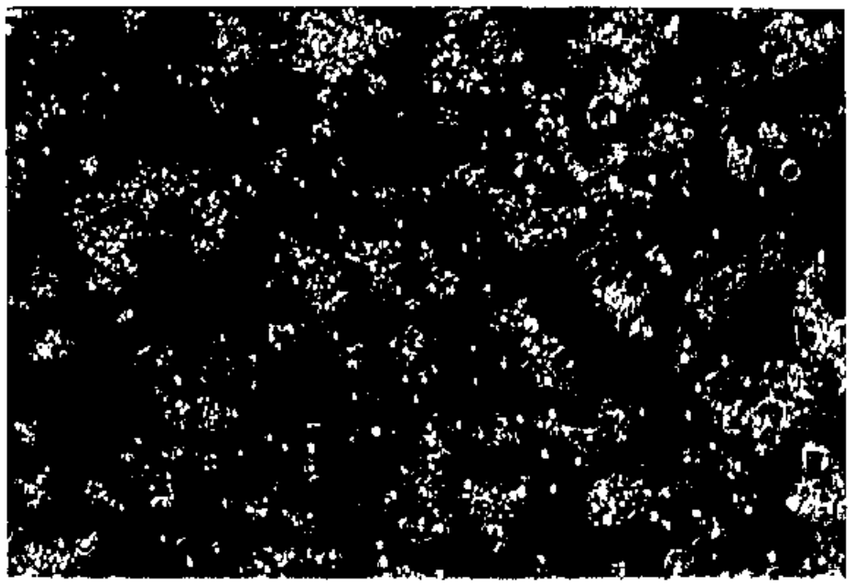
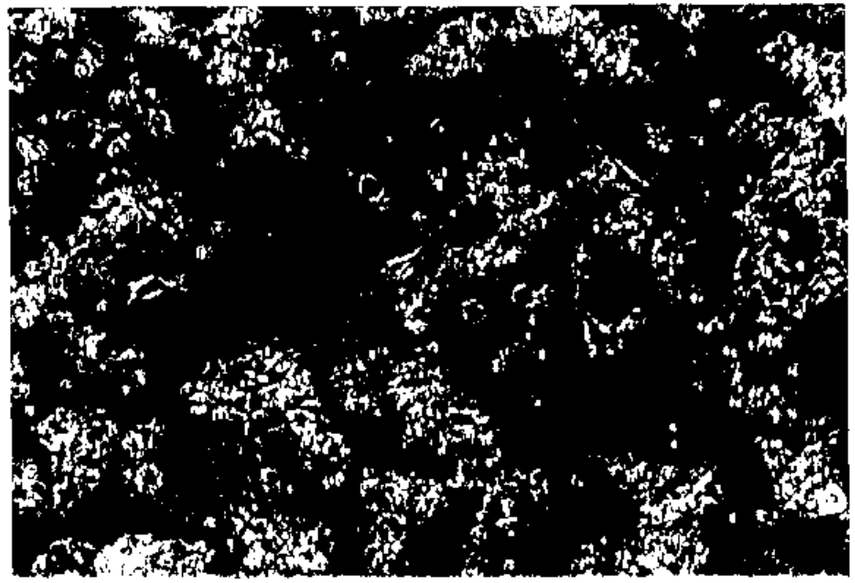
Na Auger
Ti 2p
clean glass

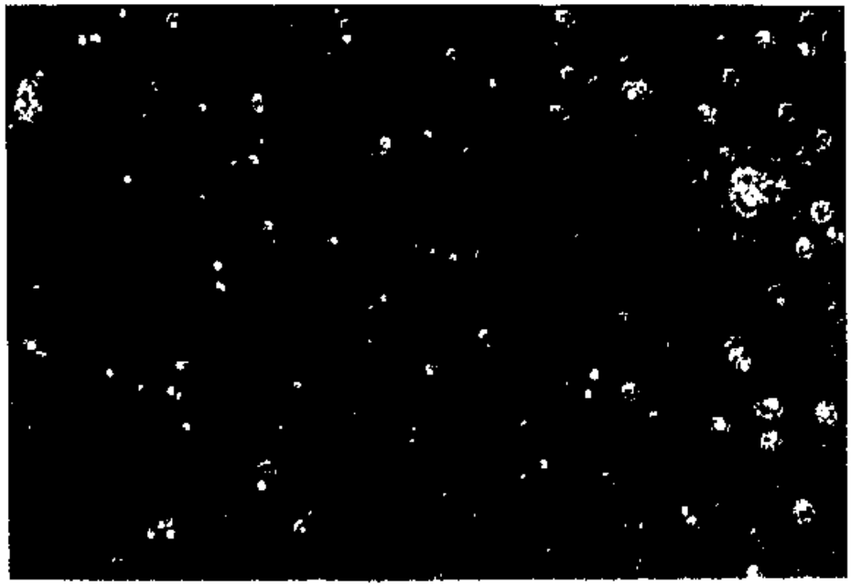
EFFECTS OF BDNF ON E22 CORTICAL CELL GROWTH



Poly-D-lysine



Poly-D-lysine + BDNF



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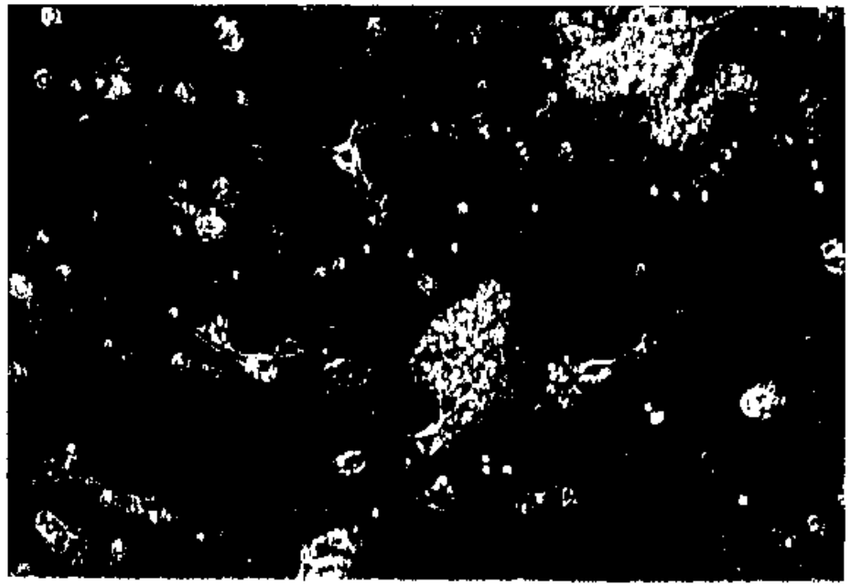
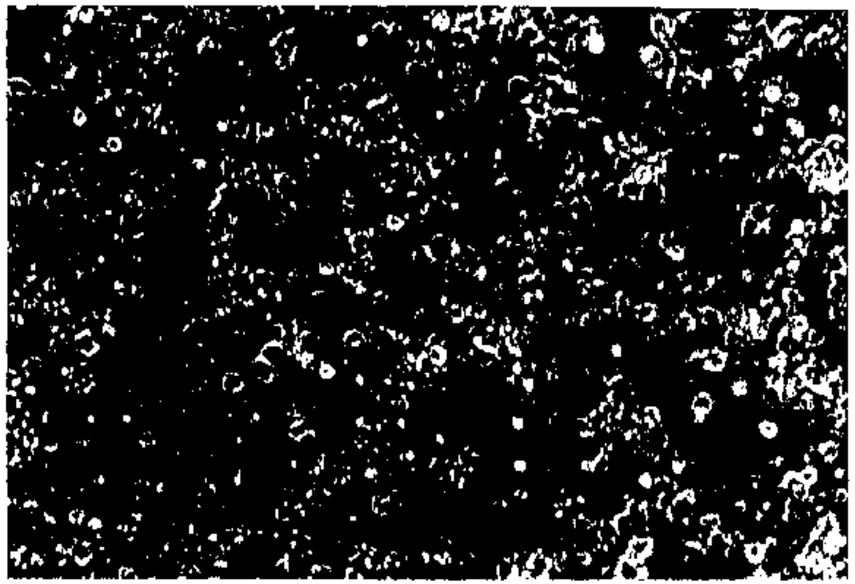


13F + BDNF

EFFECTS OF bFGF ON PN10 CELL GROWTH



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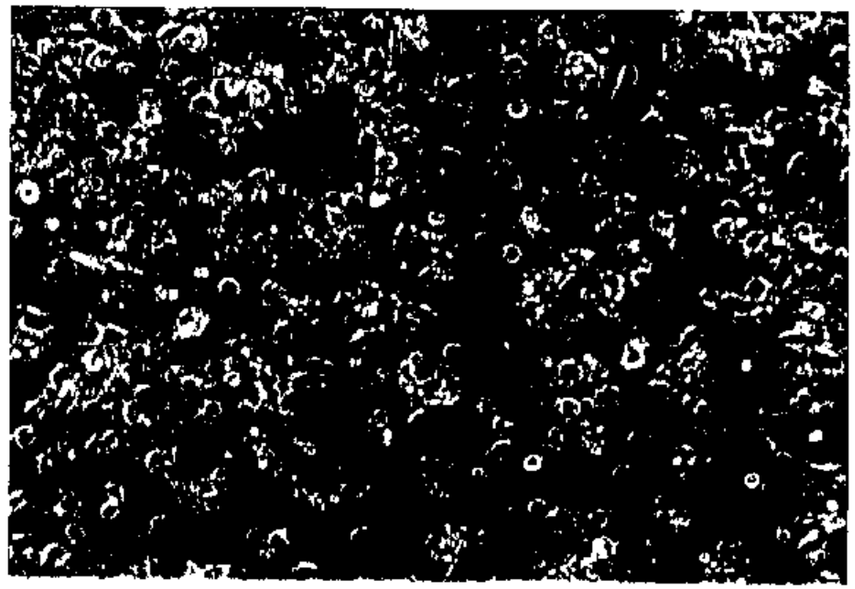
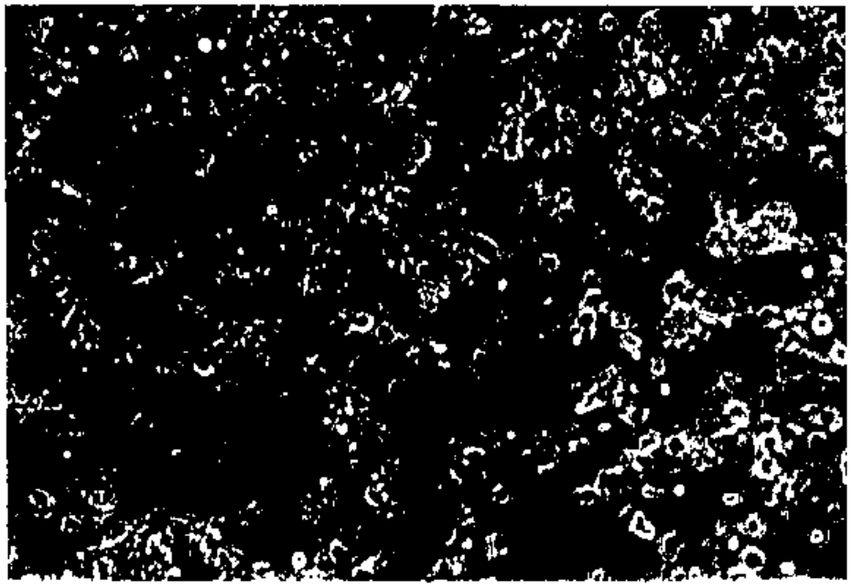


PL + bFGF



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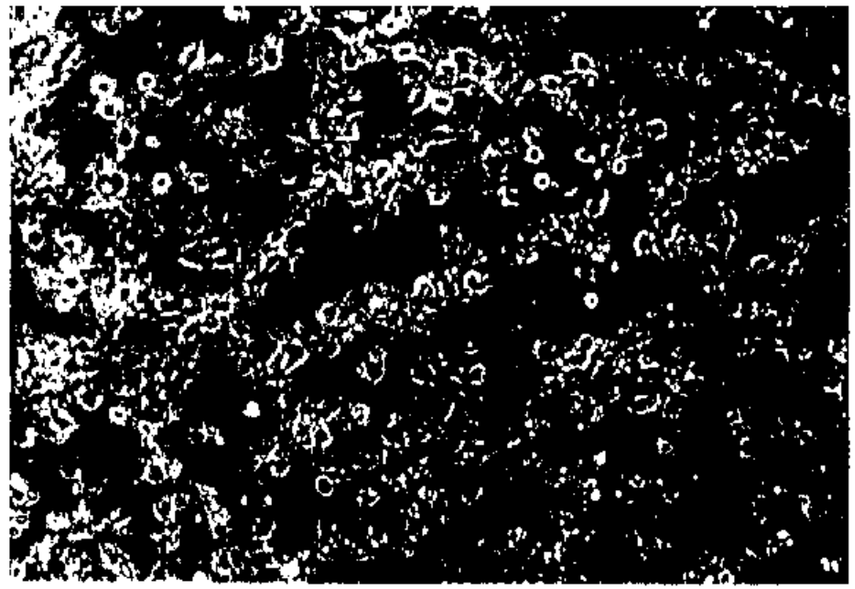
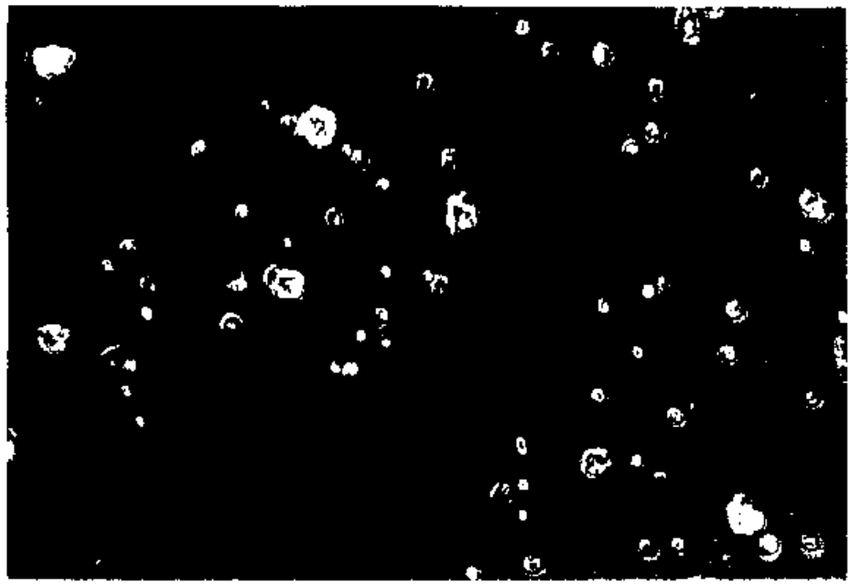


OTS + bFGF

EFFECTS OF bFGF ON E14 CORTICAL CELL GROWTH



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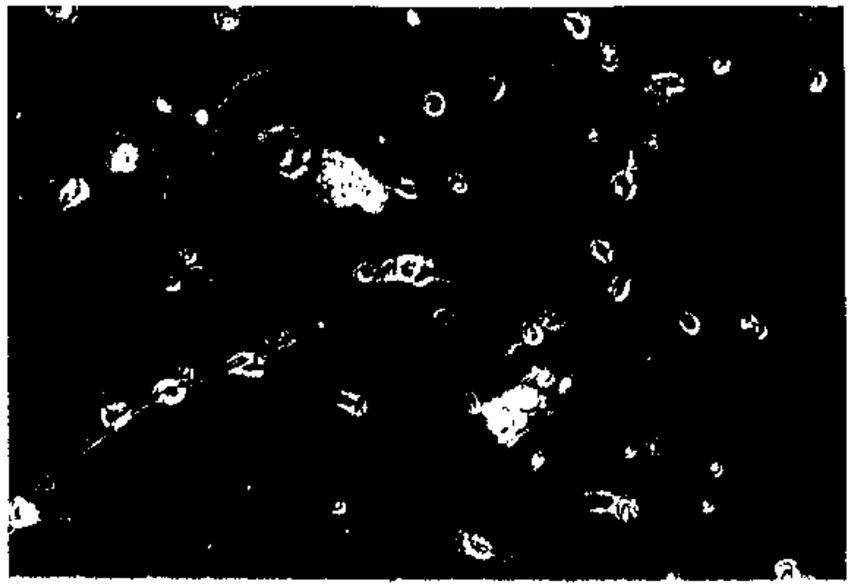
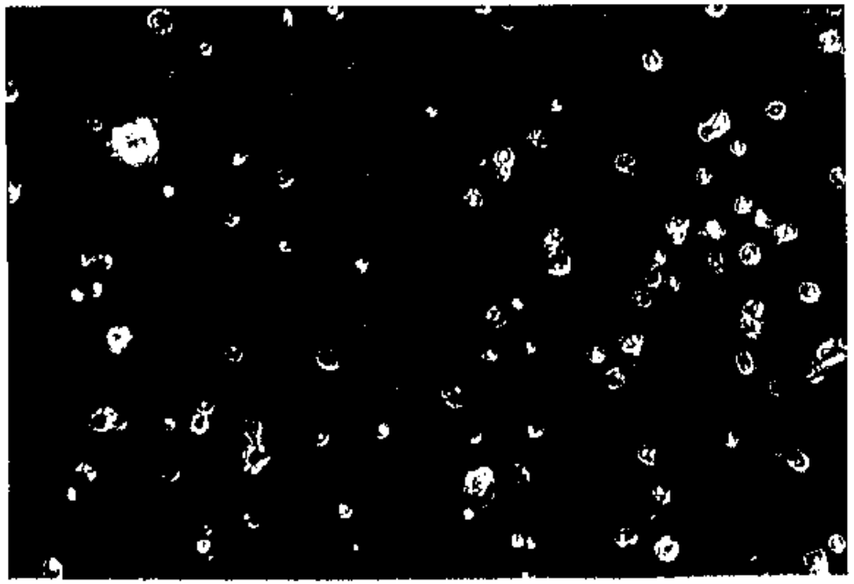


13F + bFGF



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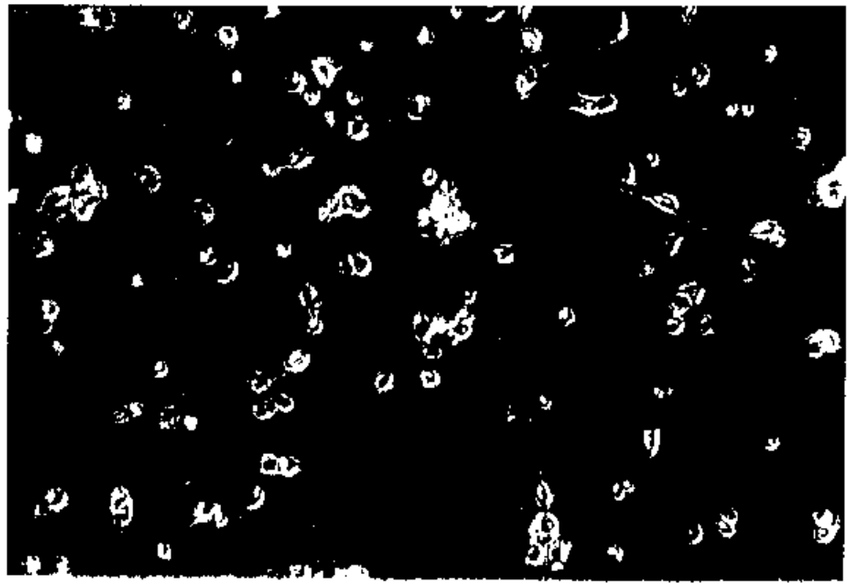

OTS + bFGF